IN THE UNITED STATES PATENT AND TRADE MARK OFFICE

MAY 8 1995 U.S. TRADEMARK OFF.

In re Application of
MARTIN J. PAGE
Serial No. 08/155,864
Filed: 23rd November 1993
For: ANTIBODY PRODUCTION

Examiner: D. ADAMS

Group Art Unit: 1806

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DECLARATION

I, JAMES SCOTT CROWE of The Wellcome Research Laboratories, The Wellcome Foundation Limited, Langley Court, South Eden Park Road, Beckenham, Kent, BR3 3BS do hereby solemnly and sincerely declare as follows:

1. I obtained the degree of B.Sc.(Hons) in Immunology from the University of Glasgow in 1980 and the degree of Ph.D. from the same university in 1983 for research into the antigenic variation in cyclically transmitted African trypanosomes. I have been employed by The Wellcome Foundation Limited since October 1983 working on various projects in the field of molecular and cell biology and since 1988 my work has been concerned with the therapeutic use of antibodies. I was appointed to the position of Senior Research Scientist in the Department of Cell Biology of The Wellcome Foundation in 1990 and I am currently head of the Antibody Engineering Group within that department. The main focus of research within my group is the application of monoclonal antibodies for human therapy. I have been programme leader for eleven monoclonal antibodies through the research phase and a number of these antibodies are now undergoing clinical evaluation, including a humanised anti-CD4 monoclonal antibody.

2. I am familiar with the contents of United States Patent Application Serial No. 08/155,864 including the new claim to be submitted for consideration by the Examiner in the Amendment

filed 15th September 1994. This application is a continuation of United States Patent Application Serial No. 08/046,893 and I have considered the reasons given by the Examiner for rejecting this preceding application as set out in the Office Action mailed 23rd June 1993 (Paper No. 14). I have also read the Declaration by Dr. Robert Lifely dated 6th April 1994 filed in support of this application.

3. THE claim now submitted for examination in this application relates to an improvement in a method for treating a mammal suffering from a disease or disorder by administering a glycosylated human or altered antibody wherein said antibody is effective in treating said disease or disorder in said mammal. More specifically, the improvement comprises administering a therapeutically effective amount of a CHO-glycosylated form of the antibody. As explained in more detail below, it has now been found that not only are CHO-glycosylated antibodies effective therapeutically in man but the nature of CHO-glycosylation is such that antibodies produced in CHO cells are particularly suited for therapeutic use in man. Antibodies are typically glycosylated on the Fc region so that this is a general effect unrelated to the antigen binding specificity of the antibody. The claim now presented emphasises the fact that the present invention is a generic invention and is not limited to the use of specific antibodies to treat specific conditions.

4. AS indicated by Dr. Lifely in his declaration, CAMPATH 1H is a humanised IgG_1 monoclonal antibody directed against the CDw52 antigen. The antibody was developed by workers at the MRC Laboratory of Molecular Biology at Cambridge and the Department of Pathology at the University of Cambridge by engineering a rat myeloma cell line and this is the form in which the antibody first came to Wellcome in about 1988. At that time, I held the position of Head of Immunochemistry within the Department of Molecular Biology at Wellcome and I became Campath 1H Programme Leader. In this capacity I initiated discussions as to how the yield of the antibody might be improved over that which could be

obtained from the cell lines developed at Cambridge. Wellcome already had considerable expertise in the production of proteins in mammalian cells, in particular CHO cells. It was decided to try to improve the yield of the antibody by engineering a CHO cell line by transfecting it with an expression construct including DNA encoding CAMPATH-1H heavy and light chains.

5. AT that time, I knew that glycosylation of secreted proteins in mammalian cells was both tissue specific and species specific, in other words the nature of the carbohydrate residues in terms of the sugars and the way in which they were linked depended both on the species from which the cell producing the protein was derived and on the tissue from which that cell was derived. I also knew that the nature of the glycosylation on an antibody affected its biological activity, particularly in terms of its ability to recruit effector mechanisms and its half life in the circulation. In this connection I would point out that the papers by Nose and Wigzell, Proc. Natl. Acad. Sci., (USA), 80, 6632-6635 (1983) and Leatherbarrow *et al*, Molecular Immunology, 22(4), 407-415 (1985) and the review by Rademacher *et al*, Ann. Rev. Biochem., 57, 785-838 (1988) mentioned by Dr. Lifely in his declaration were all available before the priority date of the basic British patent application from which the present application claims priority and are representative of the state of the art at that time. Of particular relevance is the section of the Rademacher *et al* reference entitled "Species-Specific and Tissue-Specific N-Glycosylation-Glycotypes" at pages 797 to 801.

6. IT is also relevant to point out that immunoglobulin glycosylation differs from that on other secreted proteins in significant respects. Thus, although the conserved oligosaccharide chains on IgG heavy chains are biantennary complex glycans which is a common structural motif in other glycoproteins, IgG oligosaccharides differ from other glycoproteins in two important respects. The first is the very high frequency of a core-linked fucose moiety and the second is the virtual absence of N-acetyl-neuraminic acid (NANA) in

conserved C_H2 glycans. Accordingly, it could not be assumed from the fact that other glycoproteins such as t-PA produced in CHO cells had proved to be effective therapeutically that antibodies produced in this host would also be effective.

7. MONOCLONAL antibodies had hitherto generally been produced from either rat or mouse lymphoid cells. What was now being proposed was to produce CAMPATH-1H in a cell of a different species (hamster) and of a very different type indeed (ovary). I had little doubt that the necessary engineering could be carried out to construct a CHO cell line which would produce polypeptide chains having the amino acid sequence of CAMPATH-1H. However, even assuming that the chains assembled correctly to produce an antibody like molecule, I expected that the glycosylation would be different from that on the antibody produced in rat myeloma cells and it was a major concern to me that this difference in glycosylation would have an adverse effect on the biological activity of the antibody *in vivo* and hence on its therapeutic effectiveness. Not only were the Chinese hamster ovary cells from a different species of animal and a different tissue than had been used previously for production of the antibody, but the ovary is not an organ that naturally produces immunoglobulins. It was, therefore, unpredictable whether glycosylation of antibodies by a CHO cell would result in a therapeutically functional antibody.

8. MY doubts as to whether CAMPATH-1H produced in CHO cells would be effective therapeutically led me to ensure that the company had other ways of producing the antibody should the CHO derived material prove to be ineffective. Accordingly, at the same time as the inventor of the present application Dr. Martin Page was producing constructs for the production of CAMPATH-1H in CHO cells, I was working on the production of improved rat myeloma cell lines which produced higher yields of the antibody. A major reason for this duplication of effort was the doubts that I have mentioned above over whether CHO derived CAMPATH-1H would be therapeutically effective when administered to man.

9. ONCE CHO derived CAMPATH-1H had been produced, its activity was investigated. In collaboration with my colleagues, I investigated the *in vitro* activity of CHO derived CAMPATH-1H in terms of ADCC and complement lysis in comparison to rat myeloma derived material and we concluded that CHO derived material was active in mediating both ADCC and complement lysis (see Crowe *et al.*, Clin. Exp. Immunol., 87, 105-110 (1992)). However, the most important results were those for the therapeutic effectiveness of the CHO derived antibody in man. I was surprised, albeit pleasantly surprised, when it was found that CAMPATH-1H produced in CHO cells retained therapeutic effectiveness in man. I think that this result was quite unpredictable based on the level of knowledge in the art at the time and it was not a result that I personally predicted.

10. THE first results on the therapeutic effectiveness of CHO derived CAMPATH-1H are given in the patent application (latter part of Example 5) and this result has subsequently been confirmed and extended. Publications relating to the therapeutic use of CHO derived CAMPATH-1H are referred to by Dr. Lifely in paragraphs 9 to 11 of his declaration. The present application also refers to the production of a humanised anti-CD4 antibody in CHO cells and we now have some data on the therapeutic use of this antibody in man.

11. A humanised (CDR-grafted) anti-CD4 antibody produced in CHO cells and thus having glycosylation characteristic of CHO cells has been used at Addenbrooke's Hospital in Cambridge by workers from the Department of Medicine and the Department of Pathology of the University of Cambridge as an adjunct to CAMPATH-1H in the treatment of systemic vasculitis. The clinician mainly involved in this work from the Department of Medicine has been Dr. C. Martin Lockwood. The work was supported in part by Wellcome and I was aware of the work as it progressed. A preliminary report of the work was published in the journal The Lancet in June 1993 (Lockwood et al, The Lancet, 341, 1620-1622 (1993)) and the treatment of 4 patients is described (see Table on page 1621).

The Patient 1 was treated before the CHO glycosylated humanised anti-CD4 antibody became available and he was treated with a rat anti CD4 antibody purified directly from the relevant hybridoma cell line. Patients 2 and 4 received CAMPATH-1H and the humanised CHO-glycosylated anti-CD4 antibody. Patient 3 received only CAMPATH-1H. I should point out that the Lockwood et al paper refers to the anti-CD4 antibody administered to Patients 2 and 4 simply as "a humanised monoclonal anti-CD4 antibody" but I can state from my own knowledge that the antibody was produced in CHO cells. More details of this work are included in a paper recently submitted for publication to the New England Journal of Medicine a copy of which I now provide as Exhibit "JSC 1". The main author of this paper is Dr. Lockwood and details are given of the treatment of 11 Patients of whom 8 received anti-CD4 antibody as well as CAMPATH-1H. Patients 1 to 4 are the same as the patients referred to in the Lockwood et al paper in The Lancet. Thus Patient 1 received rat anti-CD4 antibody and the other patients received CHO-glycosylated humanised anti-CD4 antibody. Lockwood et al in their paper in The Lancet conclude that:

> "Experimental studies have shown that combination therapy can give better tolerance than either mAb used alone, and better control of the progression of an established autoimmune response." (page 1621 right hand column)

In the paper submitted to the New England Journal of Medicine, the authors state:

> "We describe in this paper the successful application of humanised monoclonal antibody therapy for four of five patients with intractable T cell associated vasculitis and five of six patients with ANCA associated diseases treated during the five year period 1989-1994. We conclude that substantial benefit may be obtained in both these categories of patients and that monoclonal antibody therapy should be considered for the routine management of patients with vasculitis intolerant of, or refractory to,

conventional treatment". (page 8)

Later in the report, the authors say:

> "Our current policy is to use CAMPATH-1H as the first therapeutic agent and to use anti-CD4 as adjunctive therapy if CAMPATH-1H proves to have only a temporary effect". (page 35)

The work reported in the paper in The Lancet and the unpublished report show that both CAMPATH-1H and the humanised anti-CD4 antibody have a therapeutic effect in man and both of these antibodies were produced in CHO cells and thus have the glycosylation characteristic of CHO cells.

12. OTHER companies are also now developing CHO derived antibodies for therapeutic use. Much of the information about these products remains proprietary although the company IDEC Pharmaceuticals Corporation is entering Phase II clinical trials with a CHO derived anti-CD4 antibody. The fact that the product is entering Phase II trials means that encouraging results must have been demonstrated in man. IDEC have also published results of tests on a chimeric mouse/human anti-CD20 antibody produced in CHO cells in a non-human primate (macaque cynomolgus monkeys) (Reff et al, Blood, 83, 435-445 (1994)). The antibody showed activity in vivo in depletion of B cells in peripheral lymph nodes and bone marrow and no toxicity was observed. WO 94/02175 (ICOS Corporation/University of Washington) also demonstrates that a humanised anti-CD18 antibody produced in CHO cells showed equivalent activity in an EAE model of inflammatory disease in macaques to the parent murine antibody. All of these results strongly suggest, as I would have expected once the therapeutic effectiveness of CHO-derived CAMPATH-1H had been demonstrated, that therapeutic effectiveness of CHO derived antibodies in vivo is a general effect and is not confined to the specific antibody CAMPATH-1H.

13. ONCE CHO derived CAMPATH-1H had been produced, it was found that the glycosylation introduced by CHO cells is indeed different from that introduced by rat myeloma cells and

experiments demonstrating these differences are presented by Dr. Lifely in his declaration. It was also found after antibodies had been produced in CHO cells that the glycosylation given to antibodies by CHO cells is particularly advantageous for therapeutic use of the antibody in humans. This was a wholly unexpected and unpredictable result.

14. THUS, in a paper by Borrebaeck *et al.*, Immunology Today, 14(10), 477-479 (1993), reference is made to a possible adverse effect of the glycosylation of antibodies produced in mouse cells when these antibodies are subsequently used in human therapy. The authors say:

> "*Recently, Galili and co-workers described the presence of naturally occurring anti-Galα1-3Gal antibodies in the human population and in old world monkeys...but not in other mammalian species...We propose that the short serum half-life and thus poor clinical efficacy of mouse monoclonal antibodies is related to an endogenous Galα1-3Gal glycosylation of these molecules.*" (page 477).

To test this hypothesis the authors looked at the occurrence of this type of glycosylation on a selection of mouse and human antibodies and they go on to say:

> "*As can be seen in Table 1, the majority of the [mouse] antibodies contained Galα1-3Gal residues and it is worth noting that several of the clinically most relevant antibodies, for example 17-1A, 19-9 and HMFG-1 were particularly heavily glycosylated. The endogenous glycosylation found on mouse mAbs will immediately result in an immunocomplex formation when these antibodies are introduced into the circulation of human patients. In contrast, human mAbs produced from Epstein-Barr virus (EBV) infected human peripheral blood mononuclear cells do not show any*

signs of Galα1-3Gal-containing residues (Table 1). Furthermore, human mAbs derived from hybridomas formed using a human-mouse heteromyeloma fusion partner did not, except in one case, react with human anti-Gal antibodies." (page 477).

15. THE authors conclude:

"*How do we then overcome this immunological barrier to successful antibody-dependent immuno-therapy? One possible solution would be to clone the mouse genes coding for the heavy and light chains of the mAb and express these genes in a host cell that does not have an active α 1,3-galactosyltransferase. The lack of this enzyme activity prevents the addition of Galα1-3Gal residues to all proteins produced. The above table also points to the importance of characterising the glycosylation pattern of the expression host of choice, before using that host to express recombinant therapeutic antibodies.*" (page 478).

The nature of the glycosylation of an antibody is, of course, dependent on the host cell and not on the antibody itself so that the same type of glycosylation pattern including Galα1-3Gal residues would be expected to occur whether the antibody being produced in a mouse cell is a mouse antibody or a recombinant antibody such as a chimeric or a humanised antibody.

16. THE differences that were found between the glycosylation of CAMPATH-1H produced in CHO cells and CAMPATH-1H produced in rat myeloma cells proved very interesting in the light of the above findings about Galα1-3Gal residues. Glycosylation was analysed by digestion of CAMPATH-1H with *Achromobacter* protease I (Lys-C) which cleaves selectively on the COOH-terminal side of lysine but does not cleave at arginine residues. The glycosylated peptide from a Lys-C digest of CAMPATH-1H encompasses residues 293 to 321 of the heavy chain. The

glycosylated peptide of CAMPATH-1H derived from CHO cells and NSO rat myeloma cells was analysed by the technique of electrospray mass spectometry (ES-MS) and I now provide as Exhibit "JSC 2" a copy of the ES-MS spectrum of the glycosylated peptide of CHO-derived CAMPATH-1H. The mass spectrum is shown from m/z 1000 to 2000, split into two ranges. Multiply charged ions which correspond to different glycoforms of the glycopeptide occur at m/z 1228, 1269 and 1311 (+4 charge state) and at m/z 1637, 1691 and 1745 (+3 charge state). Ions corresponding to glycoforms are indicated as A, B and C and peaks in the spectrum not specifically labelled as corresponding to the glycosylated peptide are due to other coeluting peptides. It should be noted that there is an apparent discrepancy in abundance between the peaks marked C at m/z 1311 and 1745. This results from the fact that the peak at 1311 appears more abundant because part of the signal is due to a coeluting peptide corresponding to residues 223-247 of the heavy chain. The +3 charge envelope is a more accurate indicator of the abundance of the various glycoforms.

17. THE ES-MS spectrum does not provide direct information about the carbohydrate structures of the glycopeptides. However protein glycosylation follows standard patterns and it is reasonable to propose carbohydrate structures for the glycopeptides based on the expected motifs and the molecular weight of each peak. I now provide as Exhibit "JSC3" proposed structures for the carbohydrate portion of the glycopeptides corresponding to peaks A, B and C. These follow the expected pattern for an IgG and it should be noted that in the case of structure B the location of the galactose residue cannot be determined. I refer to these structures as "proposed" since they have been inferred from molecular weight rather than proved directly by analysis. However, I believe that these inferences are well founded and a high level of confidence can be placed in these structures.

18. I now provide as Exhibit "JSC4" the ES-MS spectrum for the glycopeptide of CAMPATH-1H produced in NSO rat myeloma cells.

The same three major glycoforms appear here as well and the peaks are again labelled A, B and C. However, an interesting feature is that two additional glycoforms occur with peaks at higher m/z values relative to the other ions in the mass spectrum. These peaks are labelled D and E and they differ in mass from the other glycosylated peaks by the addition of one or two hexose residues respectively. I now provide as Exhibit "JSC5" proposed structure for peaks D and E.

19. PEAKS D and E are not as abundant as the other glycoforms which also occur in CHO-derived CAMPATH-1H, but they are very significant since they can be inferred to represent structures which include the immunogenic Galα1-3Gal epitope discussed above. Structures including this epitope are absent from CHO-derived CAMPATH-1H. Accordingly because of the absence of Galα1-3Gal residues and the similar ratios of the three major species seen in ES-MS, antibodies produced in CHO cells show significant similarities to antibodies produced in human cells and are particularly suited for therapeutic use in man. As I have already noted this is a surprising and wholly unpredictable result.

20. MOST of the data that I have referred to above on the therapeutic effectiveness of CHO derived antibodies and all of the data on specific glycosylation patterns comes from the antibody CAMPATH-1H. Antibodies are typically glycosylated on the Fc region, more specifically the CH2 domain. Therefore, while glycosylation differences would not have been expected to affect antigen binding, they would have been expected to affect effector functions and *in vivo* half life. The concerns that I had before antibodies were produced in CHO cells about the likely therapeutic effectiveness of such antibodies were, therefore, general concerns which had to do with effector functions and the way in which antibodies are cleared from the system when administered to humans. The concerns were thus not specific to any particular antibody and applied to the way in which the body deals with antibodies and the effect of particular types of

glycosylation. Since the effector functions and clearance mechanisms are not antibody-specific but instead are universally employed by the body for all antibodies, once it was established that CHO glycosylated CAMPATH-1H is therapeutically effective, then it was fully expected that other therapeutically effective antibodies would retain their effectiveness when expressed in CHO cells.

21. THE absence of Galα1-3Gal residues in the glycosylation of CAMPATH-1H can now be explained by work on the enzymes which effect glycosylation. The enzyme responsible for the Galα1-3Gal residue is α1,3-galactosyltransferase. Smith *et al* (J. Biol. Chem., 265, 6225-6234 (1990)) noted that

> *"CHO cells synthesize Asn-linked oligosaccharides that contain poly-N-acetyl-lactosamine which have two possible terminal sequences, namely Galß1,4-GlcNAc-R and NeuAcα2,3 Galß1,4GlcNAc-R"*

i.e. they do not include the Galα1-3Gal residue. They then transfected CHO cells with DNA encoding a murine α1,3-galactosyl transferase and found that glycopeptides from the transfected cell line but not the parental cell line included terminal Galα1-3Gal linkages. This strongly suggests that CHO cells lack a functional α1,3-galactosyl transferase. Ashford *et al.*, J. Biol. Chem. 268, 3260-3267 (1993) looked at glycosylation of recombinant rat and human soluble CD4 variants expressed in CHO cells. They note that

> *"many other investigators have examined the glycosylation of recombinant glycoproteins expressed in CHO cells and found no evidence of terminal α1,3-galactose residues."* (page 5263)

They conclude that the gene must be present in CHO cells but not expressed and they claim to be the first to observe Galα1-3Gal

structures in glycoproteins expressed in CHO cells, presumably as a result of the latent α1,3-galactosyltransferase gene being activated by an artefact of the production of their engineered cell line. Ashford *et al* also note that the human equivalent of the murine and bovine α-galactosyltransferase gene appears to be present as a non-functional pseudogene as a result of frame shift and nonsense mutations. Thus it can be concluded that human and CHO glycosylated antibodies are surprisingly similar as a result of the absence of Galα1-3Gal residues in the glycosylation and this is an effect which will apply to all CHO cell derived antibodies.

22. THE Examiner has drawn attention to a paper published earlier this year by Emery and Adair entitled "Humanised monoclonal antibodies for therapeutic applications" (Exp. Opin. Invest. Drugs, 3(3), 241-251 (1994)) and in particular comments made by the authors on the effect of the glycosylation of therapeutic antibodies. Before dealing specifically with the question of glycosylation, I would like to make two general comments about this article. Firstly, it should be noted that the authors take a very positive view of the future of antibody therapy and in the first paragraph of the article (page 241) they refer to "a new optimism concerning the use of antibodies as therapeutic agents". In addition, in discussing Table 1, the authors comment that this "shows the range of biotechnology and established pharmaceutical companies which are actively involved in development of humanised therapeutic antibodies " (page 246). Secondly, in my opinion, the implication of the article that only humanised antibodies are suitable as therapeutic agents is not correct. Thus the authors say:

> "*Humanised (or human) MAbs have, in many people's view, replaced both rodent MAbs and simple chimeric..... antibodies as the basis for antibody therapeutics.*" (page 241, emphasis added).

This may be the author's view but it is not mine. The only

antibody currently licensed for therapeutic use (OKT3) is a rodent antibody and a number of rodent and chimeric antibodies are in development by different pharmaceutical companies. By way of example, my own company is developing the mouse monoclonal antibody 17-1A (now known as PANOREX) for the treatment of colorectal cancer in an adjuvant setting. The results of a Phase III clinical trial with this antibody in Germany were published recently in the Lancet (Reithmuller *et al*, The Lancet, 343, 1177-1183 (1994)) and the conclusion was, in effect, that PANOREX was as effective in terms of mortality reduction as the best currently available treatments but with much fewer side effects and less toxicity.

23. THE views of Emery and Adair also need to be viewed against the background that they work for the company Scotgen which specialises in the process of humanising antibodies and this was a paper concerned specifically with humanised antibodies. In my opinion, a more balanced view is that there is a place in therapy for all of rodent antibodies, chimeric antibodies and humanised antibodies. Furthermore, although humanised antibodies may be perceived to have advantages in some situations over rodent antibodies and chimeric antibodies, this is not to say that rodent antibodies and chimeric antibodies are ineffective as the example of PANOREX demonstrates.

24. ON the question of glycosylation, Emery and Adair state as follows:

> *"When considering the expression strategies for whole IgG antibodies, it is important to remember that they are glycoproteins. The carbohydrate polymer, attached to the CH2 domain, is believed to be important for effector functions (such as antibody dependent cellular toxicity (ADCC) and complement mediated cytolysis (ADCMC)), structural integrity and a lengthy, in vivo, serum half-life....Presently, the preferred means for the production of whole antibodies*

are mammalian cell systems using NSO, SP2/O (myeloma) or CHO host cells transfected with expression plasmids containing the appropriate antibody genes. However, at present, there is insufficient data to determine whether subtle variation of the carbohydrate structure and residue content (which may be inherent to a particular type of mammalian host cell) evokes significant changes to an antibody's physical and biochemical properties" (page 247).

25. I have a number of comments on this passage. Firstly, although written several years after the events that I have mentioned above when I initiated discussions as to how the yield of CAMPATH-1H could be increased, the passage emphasises the effect of glycosylation on certain aspects of the activity of antibodies and confirms that I had every reason to be concerned that changing the nature of the glycosylation might have an adverse effect on the therapeutic effectiveness of the antibody. I have already explained above why the results which have been obtained to date in the therapeutic use of antibodies produced in CHO cells means that it can now be fully expected that other therapeutically effective antibodies would retain their effectiveness when expressed in CHO cells. In addition, as I have also explained, the evidence is that there will be differences between the glycosylation produced by different mammalian cells but that, because of the absence of Galα1-3 Gal structures in the glycosylation, CHO glycosylated antibodies may be more suitable for therapeutic use than antibodies produced in other mammalian host cells such as mouse cells.

26. I have referred extensively above to the antibody CAMPATH-1H and at the time that Dr. Lifely made his declaration this antibody was under development by Wellcome. For the sake of completeness, I should mention that Wellcome made a public announcement on 26th September 1994 that the company had ceased clinical development of CAMPATH-1H as they believe that the compound is not likely to have adequate commercial potential in

those conditions of prime strategic importance to Wellcome. The decision for Wellcome to terminate development of CAMPATH-1H cannot affect the data which has already accumulated to demonstrate the therapeutic effectiveness of the antibody and has no impact on the conclusions that Dr. Lifely and I have drawn from this data.

27. I further declare that all statements made herein to my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issuing thereon.

J. Scott Crowe

J. Scott Crowe

Date: 2/11/94

USSN 08/155,864
Declaration by J. Scott Crowe dated 2nd November 1994

Documents Referred to in the Declaration

EXHIBITS

JSC 1	Paper recently submitted to New England Journal of Medicine relating to antibody therapy of vasculitis
JSC 2	ES-MS spectrum of the glycosylated peptide of CHO-derived CAMPATH-1H
JSC 3	Proposed structures for the carbohydrate portion of the glycopeptides corresponding to peaks A, B and C
JSC 4	ES-MS spectrum for the glycopeptide of CAMPATH-1H produced in NSO rat myeloma cells
JSC 5	Proposed structures for the carbohydrate portion of the glycopeptides corresponding to peaks D and E

OTHER REFERENCES

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(*) Leatherbarrow *et al.*, Molecular Immunology, *22(4)*, 407-415 (1985)

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(*) = Document already referred to in Lifely declaration; further copy not provided.

JSC 1

Title: TREATMENT OF VASCULITIS WITH HUMANISED MONOCLONAL ANTIBODIES

Running Head: Humanised monoclonal antibody therapy

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ABSTRACT

Background: Conventional treatment of the systemic vasculitides, such as Wegener's granulomatosis and microscopic polyangiitis with glucocorticoids and cytotoxic agents is limited by substantial side effect and cumulative toxicity of the drugs themselves, as well as by the fact that certain patients may become refractory to this therapy. Recently the development of humanised monoclonal antilymphocyte antibodies has allowed us to explore an alternative form of immunomodulation.

Methods: Eleven patients with systemic vasculitis formed the study group. Five patients had abnormal T cell infiltrates in biopsies of affected organs, associated in four with an active vasculitis. None of the five had detectable antineutrophil cytoplasm antibodies (ANCA) and all were severely ill with vasculitis refractory to conventional therapy. The remaining six patients had histologically proven ANCA associated systemic vasculitis and were considered for monoclonal antibody therapy because conventional induction treatment was ineffective or contraindicated. All received humanised, monoclonal anti CD 52 with or without anti CD4 antibodies intravenously in doses up to 40 mg/day for up to 10 days.

Results: Complete remission of disease activity was obtained promptly in all five patients with T cell associated vasculitis, lasting from 3 to 48 months and such remissions could be achieved on subsequent occasions, after relapse, in four of five. Of the six patients with ANCA associated vasculitis, one died whilst on treatment at day 7 but in the remaining five symptoms resolved and circulating ANCA disappeared for periods ranging between 1.5 and 12 months.

Conclusion: Humanised monoclonal antilymphocyte antibodies provide an effective treatment for patients with systemic vasculitis, either refractory to or intolerant of steroids or cytotoxic agents.

Key words: Systemic vasculitis

Monoclonal antibody therapy

Wegener's granulomatosis

Microscopic polyangiitis

INTRODUCTION

The conventional treatment for patients with multisystem vasculitis, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MP), has usually combined steroids and cytotoxic agents, most frequently cyclophosphamide, in a high dose induction regimen to achieve remission, followed by a lower dose maintenance regimen to safeguard the remission long term. [1,2] This empirical approach was necessitated for two main reasons: first, because the vasculitides form a spectrum of closely related diseases, hitherto lacking specific diagnostic laboratory tests and classifiable only on a clinicohistological basis, difficulty was met when trying to segregate them as homogeneous syndromes and assign them to treatment protocols; second, because the limited understanding of the pathogenetic mechanisms underlying their evolution hampered the development of more specific therapies. Experience with these regimens for patients with WG, one of the commoner and more easily recognisable vasculitides, has shown that, whilst marked improvement might eventually be achieved in 90% of patients, the median time to achieve remission on treatment was 12 months and that relapses were frequent, occurring in up to 50% of patients during follow-up necessitating further courses of therapy.[1] Similar relapse rates were found in other series.[3]

Substantial morbidity was encountered in these patients, not only due to the disease but directly due to the treatment itself. In WG, glucocorticoid induced cataract and osteopenic fractures complicated the course of patients receiving steroids in 21% and 11% respectively, all of whom developed cushingoid features and cytotoxic drug induced female infertility or haemorrhagic cystitis were problems attendant on the use of cyclophosphamide in 57% and 43% respectively, which, related to an age and sex matched population, was further complicated by a 33 fold increased risk of the development of bladder cancer.[1] There existed considerable scope for alternative treatments which might be safer and more specific.

Recently evidence has been growing that the small vessel primary vasculitides, such as WG and MP are autoimmune diseases in which both humoral and cellular immune mechanisms may play a role in pathogenesis [4,5]. Thus circulating autoantibodies to neutrophil cytoplasm antigens (ANCA) have been described in a number of these disorders and variations in their specificity [6,7], isotype [5,9], subclass [10] and affinity [10,11] have been correlated with clinical expression of disease. More direct evidence for their involvement in pathogenesis has come from experiments which demonstrate that in vitro

ANCA may participate in the production of endothelial cell injury and so initiate a train of events leading to the development of vasculitis [12,13,14]. There is less evidence for the active participation of T cells in this process although their indirect involvement by orchestrating B cell function, is considered likely. Thus, although in certain groups of ANCA associated vasculitis patients, disease activity has been correlated with fluctuation in the levels of products of activated T cells [15,16], attempts to establish antigen specific T cell lines or clones have not been successful [17]. As well as patients with ANCA associated vasculitis, there remain other patients in whom these circulating autoantibodies are not found. Whether these patients have limited disease and are a form fruste of multisystem vasculitis or represent the consequence of a different pathogenetic mechanism is uncertain. Evidence suggesting the latter possibility comes from the identification of a small number of patients who have florid multisystem vasculitis, biopsy of which reveals lymphocytic infiltrates of T cells in and around vasculitic lesions, yet they neither have circulating ANCA nor respond to conventional immunosuppression.[18]

The demonstration that autoimmune mechanisms might be operating in the development of systemic vasculitis encouraged us to explore the application of more specific forms of therapy than the steroid and cytotoxic drugs routinely used hitherto.[19] Such an opportunity arose with the availability for clinical use of humanised monoclonal antibodies with specificity for lymphocytes, particularly encompassing T lymphocytes. This coincided with the referral of certain patients with refractory, ANCA negative, lymphocytic vasculitis. The success which we had in using monoclonal antibodies such as anti CD52 (CAMPATH 1H) and anti CD4 in the treatment of these rare patients with intractable vasculitis, a brief interim report of which appeared elsewhere [18], led us to consider their use for other patients with the more usual ANCA associated vasculitis, either as a supplement for ineffective conventional immunosuppressive drug therapy or, as an alternative to it, since in the latter circumstance conventional treatment was contraindicated because of risks from cumulative toxicity or undesirable side effects. In these patients ANCA acted as a useful disease surrogate indicating the need for treatment escalation or response to it.

We describe in this paper the successful application of humanised monoclonal antibody therapy for four of five patients with intractable T cell associated vasculitis and five of six patients with ANCA associated disease treated during the five year period 1989-1994. We conclude that substantial benefit may be obtained in both these categories of patients and that monoclonal antibody therapy should be considered for the routine management of patients with vasculitis intolerant of, or refractory to, conventional treatment.

METHODS

Monoclonal antibodies

Two human monoclonal antibodies were used in these studies. The first, anti human CD52 (CAMPATH 1H), a humanised anti lymphocyte antibody, is genetically engineered to have its rat hypervariable complementarity determining regions grafted into a human immunoglobulin framework.[20] The CD52 antigen is predominantly expressed on human lymphocytes, macrophages and monocytes but not on other cell types.[21,22] Antibodies raised against this efficiently lyse lymphocytes but not monocytes, in the presence of human complement and are known to be lympholytic in vivo probably through involvement of complement and cellular effector systems.[21] Within the

lymphocyte population the major effect of anti CD52 appears to be on T cell numbers and T cell function, with sustained depletion of both CD4+ and CD8+ subpopulations being reported after its administration in man; a more transient depletion of B cells is also seen.[23] The second monoclonal antibody, hIgG1CD4, a humanised anti CD4 antibody can interfere with the function of the CD4 antigen [18]. Before this was available, Patient 1 received the rat monoclonal IgG2bCD4 from which the humanised version was derived [19].

Antiglobulin and antiidiotype responses

Anti CD52 and anti CD4 antibodies were determined in double-capture ELISAS as previously described [24]. The anti CD52 assay was capable of detecting 2μg of polyclonal goat antihuman IgG (Fc specific, Sigma # 12136) and 10 ng/ml of monoclonal anti-idiotype antibody YID 13.9 (which recognises the anti CD52 idiotype). The rat anti CD4 assay could detect 250 ng/ml of the anti rat IgG2b mAb NORIG 7.16. Currently there is no monoclonal anti-idiotype reagent recognising human IgG1CD4.

T cell subsets

These were determined as described previously.[13]

Autoantibody assays

Both indirect immunofluorescence (IIF) assays and ELISAs were performed to detect ANCA. Initially the latter incorporated a crude extract of neutrophil cytoplasm containing the known ANCA antigens as solid phase ligand[25]. Subsequently antigen specific assays using the purified molecular species either proteinase 3 (Pr3) or myeloperoxidase (MPO) were developed. Solid phase immunoradiometric assays were used to detect circulating autoantibodies to glomerular basement membrane (GBM).[26]

ANCA assays

ANCA IIF, ANCA ELISA and anti MPO ELISA assays were performed as previously described [25,27]; ELISA results were expressed as percentage binding of a positive control (normal < 16%).

Anti Pr3 ELISA

Purified Pr3, prepared according to the method of Kao et al [26] (a kind gift of Dr M H Zhao), was diluted to 2 μg/ml in bicarbonate buffered saline pH8.4 and coated on microtitre plates (Dynatech, USA) for 16 hours at 4°C. Test sera diluted 1/50 in phosphate buffered saline (PBS) containing 1% gelatine (Sigma) and 0.1% Tween 20 (Sigma, PBS gel Tw), were added in duplicate . Binding was detected by an alkaline

phosphatase conjugated polyclonal antihuman IgG (Jackson, Pensylvania, USA) diluted 1:3000 in PBS gel Tw, followed by alkaline phosphatase substrate (Sigma) diluted 1 mg/ml in substrate buffer. Volumes were all 100 μl and subsequent incubations were for one hour at 37°C. Plates were washed three times with PBS containing 0.1% Tw 20 between stages and absorbance at 405 nm measured using a Titertek ELISA plate reader. Binding to control non antigen coated wells was subtracted from that for Pr3 coated wells and results expressed as percentage binding of a control positive WG serum. The upper limit of the normal range was taken as the mean + 2SD binding of 20 normal sera.

Anti GBM assays

These were performed as described elsewhere [26].

CASE REPORTS

(i) Patients with T cell associated vasculitis

Patient No: 1

Diagnosis: Microscopic polyangiitis (polyarteritis)

This 66 year old man developed polyarteritis when he was aged 40. The full details of

his presentation have been described previously [19]. The vasculitis involved skin, joints, muscles, sclera, pleura and pericardium. Laboratory investigations showed only a normochromic normocytic anaemia and evidence of an acute phase response. Biopsies confirmed an arteritis, however, and atypically there was an infiltrate of T lymphocytes, the majority of which were CD8+ cells. Prednisone, cyclophosphamide, azathioprine, plasma exchange, cyclosporin A and high dose intravenous immunoglobulin therapy had all proved ineffective or had to be withdrawn because of side effects. By the time he was considered for monoclonal antibody therapy, in November 1988, he was severely obtunded with multi system disease.

Patient No: 2

Diagnosis: Microscopic polyangiitis

This 21 year old woman presented at the age of 14 with polyangiitis which produced fever, sweats, rash, pericarditis, pleurisy and acalculous cholecystitis. Laboratory investigations showed a normochromic normocytic anaemia, neutrophil leucocytosis, thrombocythaemia and acute phase response with an elevated C-reative protein (CRP). Biopsies of skin, liver and kidney whilst on immunosuppressive drugs showed no specific significant abnormalities. Treatment with steroids, even with prednisolone up

to 40 mg/day, produced only a limited response. Over the next four years, despite azathioprine, methotrexate, plasmapheresis, cyclophosphamide, cyclosporin A and high dose pooled intravenous immunoglobulin therapy, the arthropathy became crippling and sufficiently severe that this and the right subcostal pain required opiate analgesia, which only gave partial relief. Synovial biopsy revealed an acute exudative and proliferative synovitis with prominent lymphocytic infilltration together with perivascular cuffing. The majority of these cells were of CD4 phenotype together with an admixture of CD8 positive cells and B cells. At the time she was referred for monoclonal antibody therapy, in December 1989, she had been bed-bound for eight months.

Patient No: 3

Diagnosis: Sjögren's syndrome with renal tubular acidosis complicated by systemic vasculitis

This 43 year old woman presented with renal tubular acidosis and Sjögren's syndrome at the age of 37. Subsequently she developed disabling arthralgias, bouts of pleurisy with dyspnoea, accompanied by radiological evidence of pulmonary infiltrates and neurological abnormalities, which included transient episodes of monocular blindness, paraesthesia and numbness. Renal and lung function progressively deteriorated, see Fig

l, and there were also recurrent bouts of pancreatitis. Salient abnormal investigations included evidence of an acute phase response, diminished creatinine clearance and reduced pulmonary gas transfer. Retinal angiography disclosed capillary microaneurysms. Renal biopsy showed a marked interstitial nephritis with an infiltrate of CD8+ cells; transbronchial biopsy showed haemosiderin laden macrophages and perivascular cuffing with CD8+ cells; synovial biopsy showed a chronic inflammatory infiltrate and skin biopsy showed mild perivascular infiltrate with polymorphs and lymphocytes. This patient required 25 hospital admissions in the four year course of her illness during which prednisolone (up to 60 mg/day), cyclophosphamide, azathioprine and cyclosporin A had been used without successful effect.

Patient No: 4

Diagnosis: Behçets disease/polyangiitis

This 55 year old man presented in 1987 with vasculitis which had features of both Behçets disease and microscopic polyangiitis. He had a vasculitic rash, thrombophlebitis and arthritis, lung involvement with dyspnoea and pulmonary infiltrates as well as renal involvement with haematuria and proteinuria. Investigations showed a normochromic, normocytic anaemia, thrombocytopenia of 80,000/cu mm and an acute

phase response. The creatinine clearance was 71 ml/min and 24 hr urine protein 1.0 g. Renal biopsy revealed a healed focal segmental glomerulonephritis with evidence of old crescent formation without immune deposits. Transbronchial biopsy showed a leucocytoclastic vasculitis. Vein biopsy of a new phlebitic lesion produced evidence of intimal hyperplasia with adventitial haemosiderin deposition suggestive of previous red cell extravasation and a CD8+ lymphocytic infiltrate in the vein wall. Skin biopsy showed a mild perivascular infiltrate with acute and chronic inflammatory cells. He failed to respond to therapy which included prednisolone (up to 100 mg/day), cyclophosphamide, azathioprine, plasmapheresis, intravenous immunoglobulin (high dose), and an incomplete course of total lymph node irradiation as well as monoclonal antibody therapy with OK T3.

Patient No: 5

Diagnosis: Wegener's granulomatosis

This 30 year old woman developed a persistent oral antral fistula after failed intranasal antrostomy for recurrent sinusitis at the age of 16, in 1979. Biopsies of the oral lesions did not yield a histological diagnosis. In 1981 she developed episodes of weakness, night sweats and headaches and intermittent iritis of the left eye. She was given a full

course of radiotherapy to the palatal lesion, followed by a full systemic course of chemotherapy. Although responding initially the disease again relapsed and this time the symptoms were accompanied by episcleritis and proteinuria. In 1991 she was treated with further cyclophosphamide and prednisolone, but after two months these were discontinued because of marrow suppression, which proved to be long lasting. She was then referred to Cambridge for further management. A conclusive diagnosis of Wegener's granulomatosis proved difficult to reach, possibly because of the effects of previous radiochemotherapy. Repeated attempts to obtain a tissue diagnosis only revealed evidence of chronic inflammation without any vasculitis but in one palatal biopsy, as well as on the renal biopsy, there was evidence of an unusual T cell infiltrate without any other diagnostic features, apart from there being an accompanying focal tubular atrophy in the renal biopsy. Particularly, there was no evidence of T cell gene rearrangement, nor was there any evidence of lymphoma. Other laboratory investigations failed to show leucocytosis, thrombocytosis or positive serology for ANCA. The disease progressed, with an enlarging ulcerating granulomatous maxillary sinus lesion, evidence of peripheral sensory neuropathy and increased urinary protein

excretion. There was no sustained effect of either high dose intravenous immunoglobulin or of cyclosporin A.

(ii) **Patients with ANCA associated vasculitis**

Patient No: 6

Diagnosis: Wegener's granulomatosis associated with anti Proteinase 3 (Pr3) autoantibodies

This 56 year old man presented with proteinuria in May, 1992. ANCA serology was positive for circulating anti Pr3 autoantibodies and renal biopsy showed a focal necrotising glomerulonephritis, both compatible with the diagnosis of WG. He was started on conventional therapy with cyclophosphamide 150 mg od and prednisolone 60 mg od. However, because the plasma creatinine, which was 3.4 mg/dl (307 μmol/l) at presentation, continued to rise and a short course of plasma exchange revealed that autoantibody synthesis was still active, despite two weeks of immunosuppressive drug treatment, he was referred for further treatment. An autologous isotope-labelled polymorph scan showed nasal and lung involvement consistent with WG [28], isotope EDTA glomerular filtration rate (GFR) was 5mls/min and renal biopsy showed acute necrotising glomerulonephritis with crescent formation.

Patient No: 7

Diagnosis: Rapidly progressive glomerulonephritis associated with anti Pr3 and anti glomerular basement membrane (GBM) autoantibodies.

This 66 year old man presented in August 1992 with a six month history of night sweats, cough with expectoration of white sputum and a two week history of diarrhoea with weight loss. On admission he was found to have a creatinine of 17.0 mg/dl (1547 μmol/l) and a renal biopsy showed evidence of a recent florid, uniformly crescentic glomerulonephritis with an accompanying vasculitis. Immunofluorescence showed linear IgG deposited along the GBM. Serologically he was found to have both circulating ANCA (anti Pr3) and anti-GBM antibodies. Autologous isotope labelled polymorph scan showed diffuse uptake in both lungs, together with increased uptake in the region of the right maxillary sinus, appearances which were consistent with diagnosis of WG. He was commenced on prednisolone and cyclophosphamide in conventional dosage and although this led to reduction in ANCA levels, the anti-GBM antibody synthesis was still active, even after two weeks of immunosuppression. He was then referred for monoclonal antibody treatment in the hope that because of the recency of the nephritis, and the

coincidental vasculitis. control of both of these might avoid the otherwise inevitable permanent loss of renal function.

Patient No: 8

Diagnosis: Wegener's granulomatosis associated with anti Pr3 autoantibodies

This 73 year old woman was transferred in November 1992 to Addenbrooke's hospital with a three month history of nose bleeds and a one day history of haemoptysis. She was found to be anaemic with marked chest x-ray shadowing. At presentation her plasma creatinine was 3.5 mg/dl (317 μmol/l) and her pO_2 was 59 mmHg (7.6 kPa). Serologically she had antibodies to Pr3. Because of her age. frailty and incipient risk of infection. this patient with WG was started on a combination of plasma exchange to remove the autoantibodies associated with vasculitis together with Campath 1H to control further autoantibody production.

Patient No: 9

Diagnosis: Wegener's granulomatosis associated with anti Pr3 autoantibodies

This 65 year old man was admitted to another hospital in 1989 with pneumonia and a right retrocardiac shadow seen on chest xray. He had normal renal function at that time. Bronchoscopy and biopsy of the lung lesion suggested a diagnosis of squamous

carcinoma of the lung. He was found to have renal impairment and managed conservatively. Renal failure progressed and, in September 1990, he was readmitted, whereupon a renal biopsy showed vasculitis changes compatible with a diagnosis of WG. Anti Pr3 antibodies were detected. He was treated with cyclophosphamide and prednisolone and with this his ANCA titre became negative. His chest x-rays also became normal and he remained well until January 1992. At that time he developed haemorrhagic cystitis and a small contracted bladder. During 1992 two new lesions appeared on the chest x-ray, his ANCA titre became positive and the pulmonary changes progressed despite increasing the prednisolone to 30 mg/day, a course of azathioprine, which had to be discontinued because of leucopenia, and treatment with high dose intravenous immunoglobulin. With increasing malaise and dyspnoea he was referred for further management in February 1993. Diagnostic imaging showed marked multiple bilateral ill-defined cavitating pulmonary nodules. CT of the chest showed irregular necrotic masses in both lungs, particularly affecting the upper lobes in keeping with WG. White cell scan showed marked pulmonary uptake and focal abnormalities were also seen in the left renal area. Lung biopsy showed areas of vasculitis.

Patient No: 10

Diagnosis: Microscopic polyangiitis associated with anti myeloperoxidase (MPO) antibodies

This 76 year old man had a long history of chronic obstructive airways disease with recurrent chest infections. For five years he had intermittent haemoptysis, circulating anti MPO antibodies and haematuria with proteinuria which on renal biopsy was shown to be due to a focal necrotising glomerulonephritis. Other symptoms included arthralgias and a sensory neuropathy. With a variety of treatments disease activity had fluctuated. However, after 12 months free of any symptoms and without treatment, he was admitted in August 1991 with a history of one week of pleuritic chest pain, intermittent haemoptysis for five days, with fevers and sweats for 48 hours. He had also developed arthralgias, particularly in his knees. One week before admission he had been started on prednisolone 30 mg/day by his own doctor and on admission he was treated with antibiotics and plasmapheresis. Risk of further chest infection contraindicated the use of cytotoxic agents. There was only a transient effect on ANCA levels, which continued to rise after plasmapheresis had been stopped. He was therefore considered for monoclonal antibody therapy.

Patient No: 11

Diagnosis: Microscopic polyangiitis with anti MPO antibodies

This 65 year old retired man was admitted in March 1993 with progressive symptoms due to his microscopic polyangiitis, consisting of three months worsening malaise, myalgias and arthralgias. Biopsy of the kidney, at a time when he had proteinuria and haematuria, had revealed a focul proliferative glomerulonephritis compatible with the diagnosis of microscopic polyangiitis and circulating anti MPO antibodies were present in raised titre. For six weeks he had experienced increasing dyspnoea and wheeze. He had previously failed treatment with steroids, cyclophosphamide and azathioprine. Because of cataract formation he was unwilling to receive further steroid treatment and, because of the risk of infection, was not prepared to receive further immunosuppressive drug therapy.

RESULTS

Monoclonal antibody therapy

The dates of treatment, total and individual doses of the monoclonal antibodies used are detailed in Table 1.

Patient 1 Treatment was commenced with CAMPATH 1H alone, 2 mg daily for eight days, with a striking remission of symptoms within 48 hours, although relapse occurred ten days later. Two further courses achieved only short-term improvement. However, after a further three day course, followed by treatment with a rat anti CD4 antibody on 12 consecutive days, there was complete remission of symptoms which was maintained for four years. Steroids were reduced from 20 mg daily to an adrenal replacement dose (6 mg daily). Gradually, intermittent episodes of skin lesions, similar to those present pre-treatment, occurred with increasingly troublesome frequency. Therefore a repeat course of treatment was given in March 1993. Again this controlled the manifestations of the vasculitis, but the patient died of a carcinoma of the colon in June, 1993.

Patient 2 CAMPATH 1H was started at 2mg/day for five days, then 10 mg/day for five days. Within 48 hours the arthritis subsided and analgesia could be gradually withdrawn. CRP levels became normal after the CAMPATH 1H was discontinued. Steroid therapy was lowered from 30 mg/day pre treatment so that, five months after receiving monoclonal antibody therapy, the patient's medication was prednisolone 6 mg/day. This patient's course was complicated by relapses and by the development of

an antiidiotypic antibody to the CAMPATH 1H which was first detected three months after the second successful course of antibody therapy, see Fig 2. This blocked the effect of the monoclonal antibody both in vivo and in vitro. However, when plasma exchange was carried out to lower the circulating level of this antibody in vivo and the patient treated again with a combination of CAMPATH 1H together with anti CD4, remission after this third course of therapy was again achieved; see, Fig 2. This patient has now received five courses of monoclonal antibody therapy during the three year study period, for the most part as an out-patient. Removal of the antiidiotypic antibody by plasma exchange before administration of the monoclonal antibodies has been successful for the last three treatments. Currently this patient is in remission receiving prednisolone 5 mg daily and methotrexate 10 mg weekly.

Patient 3 Treatment with CAMPATH 1H 2mg/day for five days, then 10 mg/day for five days, was well tolerated. There was subjective improvement in arthralgias and dyspnoea and objective improvement in creatinine clearance, see Fig 3. However, symptoms returned, renal function deteriorated and the lymphocyte count rose slowly towards pre-treatment values after 10 days. CAMPATH 1H was reintroduced at 40

mg/day for five days with clinical improvement again. There was a transient return of symptoms at two weeks but subsequently the arthritis subsided. Both renal and respiratory function showed improvement which was sustained over the next 30 months of follow-up, with return to within the normal range, see Fig 1. Steroid treatment was reduced and withdrawn completely after 18 months. No further hospital admissions were required and she remains in remission.

Patient 4 Treatment was given as CAMPATH 1H 2 mg/day for five days, then 10 mg/day for five days. There was striking improvement in symptoms but a relapse after three months was unresponsive to a second course of monoclonal antibody therapy. Although the number of CD4+ cells fell again after the second course of treatment, cytokine profiles showed that persistent high levels of gamma interferon were present and that there had been no effect of the monoclonal antibody therapy on their production. His disease remained active despite prednisolone 40 mg/day until he was started on thalidomide 300 mg/day, which was given in the expectation that it would control the effects of the gamma interferon [29]. With this treatment there was successful

control of the phlebitis during the next twelve months of follow-up and the prednisolone reduced to 12.5 mg daily.

Patient 5 Because of the abnormal T cell infiltrates on biopsy she was started on CAMPATH 1H, initially at 2 mg/day and subsequently increasing to 40 mg/day. With this there was improvement in the autologous isotope-labelled polymorph cell scan, which showed disappearance of the nasal and pulmonary uptake pretreatment, as well as an increase in the isotope GFR from values of 48 and 50 mls per minute in the month prior to CAMPATH therapy, to 67 and 74 mls per minute in the month post CAMPATH therapy. There was also clinical improvement as far as pain in the sinus area was concerned. However, three months later the pain recurred and inspection of the lesion indicated renewal of disease activity. On this occasion she was treated with CAMPATH 1H in association with anti CD4. Again the sinus lesion improved with lessening of the pain and decreasing of malaise. However, after two months the left maxillary antrum was worse and there was return of the tiredness, night sweats and a more marked peripheral neuropathy. It was elected therefore to try to administer the monoclonal antibody local to the antral lesion and so intra arterial injections of CAMPATH 1H were

given to the left maxillary artery. There was improvement in the patient's symptoms with marked diminution of the pain on the left side, increased mobility and disappearance of the peripheral neuropathy. At inspection during the subsequent eight months the lesion in the left maxillary antrum showed continued regression.

Patient 6 CAMPATH 1H was given at 4 mg/day for ten days. However, a further course of plasma exchange revealed that antibody synthesis still continued and so CAMPATH 1H, as additional immunosuppression, was given at 40 mg/day for seven days, after which levels fell, remaining near the normal range until discharge from hospital, see Fig 3. A repeat white cell scan was normal. Renal biopsy after treatment showed merely scarred glomeruli compressed by old fibrous crescents, without any active glomerulonephritis. Isotope GFR showed a value of 12 mls/minute: the patient thereafter required haemodialysis twice weekly and is well on follow-up. Maintenance prednisolone continued at 10 mg/day. In this patient measurements of ANCA were used as surrogates of disease activity and helped determine the treatment strategy. Although the autoimmune response was eventually controlled, irreversible destruction of glomerular architecture probably precluded substantial recovery of renal function.

Patient 7 Attempts to control anti GBM antibody synthesis were made with CAMPATH 1H at 40 mg/day for five days, followed by anti CD4 20 mg/day for five days. After the monoclonal antibody treatment, a further short course of plasma exchange showed that anti GBM antibody synthesis had ceased, thereafter levels of anti-GBM antibody fell to background and have not been detected during follow-up. The cyclophosphamide therapy was discontinued after only 18 days (an event necessitated by the development of leucopenia and thrombocytopenia). Steroids were withdrawn gradually and discontinued after three months. There was no recovery of renal function and the patient remains on renal dialysis therapy. The rapid termination of anti GBM antibody production, however, suggested that earlier intervention in the course of such an anti GBM nephritis might produce better renal recovery.

Patient 8 CAMPATH 1H was given at doses up to 40 mg/day for five days, see Table 1. With this treatment, the haemoptysis ceased, her chest x-ray cleared and her pO_2 rose. Furthermore, the rate of decline of her renal function diminished and her autoantibody titres fell. However, one week later she had a cerebrovascular accident and died suddenly. Autopsy revealed lung and renal histology compatible with WG: it was

not possible to determine what effect there had been on disease progression of the treatment started only one week earlier.

Patient 9 It was decided to escalate therapy with CAMPATH 1H at 4 mg/day increasing to 40 mg/day. One week after completing the course of treatment the chest x-ray had begun to improve and continued to improve over the period of six months follow-up. ANCA titres became negative and remained negative during follow-up whilst prednisolone was reduced from a daily dose of 40 mg to 10 mg four months later, at which time all the lung lesions had disappeared. Only a small scar remained in the region of the right mid zone, see Fig 4, and he remained well at follow-up 10 months later.

Patient 10 CAMPATH 1H given at 2 mg/day for 5 days, followed by 10 mg/day for 5 days. The ANCA titres fell over the next 6 months to become undetectable. Haemoptysis ceased and he became asymptomatic. Haematuria and proteinuria disappeared. ANCA titres became elevated again in September 1992 and haematuria became detectable again at this time. The haematuria increased in quantity over the next

four months and later was accompanied by proteinuria also. He was admitted in February 1993 with a six week history of rash and wheeze. He was given a combination treatment at this time with CAMPATH 1H up to 40 mg/day for five days, followed by anti CD4 20 mg/day for five days, see Table 1. Four days after he completed the monocional antibody therapy he developed pneumococcal pneumonia which responded to antibiotic treatment. The symptoms of rash and wheeziness disappeared, and he was still well at follow-up five months later.

Patient 11 This patient was treated with plasmapheresis and CAMPATH 1H. With that his symptoms improved, circulating anti MPO antibodies disappeared and he was discharged, to be followed up as an outpatient. He remained well for three weeks with complete resolution of arthralgias, dyspnoea, myalgias and wheeze. However, six weeks later he was readmitted with return of the same symptoms and a rising anti MPO titre. On this occasion he was given CAMPATH 1H in conjunction with anti CD4 monoclonal antibody therapy, see Fig 5. Symptomatology resolved and anti MPO antibodies disappeared by the time of discharge but there was thereafter a progressive worsening

of the arthralgias. No cause for these could be found, he remained ANCA negative and synovial biopsy showed no evidence of inflammation. Gradually the symptoms due to the arthritis subsided, disappearing completely over the next six weeks of follow-up with complete regression thereafter (for six months to present time).

Lymphocyte depletion in patients 1-4 with T cell associated vasculitis

The effect of monoclonal antibody therapy on peripheral CD4 populations is shown in Fig 6. There was substantial depletion of the CD4 cells, measured as % total circulating lymphocytes (normal range >25%). In patients 1 and 2 a rise in the proportion of CD4 cells occurred at times of relapse (R). Despite a low proportion of circulating CD4 cells systemic opportunistic infections were not seen in these patients, although patient 4 developed a candidal oesophagitis which responded to appropriate antifungal drugs.

DISCUSSION

The striking response to humanised monoclonal antibody therapy shown by the five patients with T cell associated vasculitis, which was refractory to a wide range of immunosuppressive drugs, provided strong evidence that a novel therapeutic mechanism

had been engaged by this treatment strategy. Thus these five patients had all previously received high dose steroids, alkylating agents, cyclosporin A, pooled intravenous immunoglobulin and, except for one, plasma exchange also, alone or as combination therapy with little or no effect on the progression of their disease. Monoclonal antibody therapy brought about prompt and complete resolution of disease activity in all with such remissions lasting from 3 to 48 months. The pathogenesis of their vasculitic injury was unclear but seemed to owe more to cellular than to humoral autoimmune responses, based on the presence of T cell infiltrates in the vasculitic lesions and the absence of vasculitis associated autoantibodies. Furthermore the relapses in Patient 1 and Patient 2 appeared to be accompanied by a rising proportion of CD4+ cells in the population of circulating lymphocytes. The relatively rapid nature of the clinical response to lymphocyte depletion with CAMPATH 1H, with or without CD4 blockade, which was within 48 hours in most patients, also argued that the effect was mainly through cellular mechanisms, since the longer half life of immunoglobulin would preclude such a speedy response if the disease involved a predominantly humoral pathogenesis.

A second feature, notable from our studies, was the ability to titrate the dosage of the monoclonals to produce the best effect on the patients. There was evident dose dependence shown in Patient 3 and Patient 6, as well as a synergistic requirement between the two monoclonals demonstrated in Patient 1 and Patient 11. Similar synergy has been demonstrated in an experimental model of autoimmune arthritis, wherein monoclonal antibodies, similar in specificity to those used for the patients with vasculitis, were capable of arresting an ongoing autoimmune response when used together, whereas neither alone could affect the progression (although either alone would block the induction) [30]. These data demonstrate how an understanding of the appropriate dose, duration and combination of monoclonal antibody therapies based on the experience with each individual patient can lead to the evolution of effective treatment strategies and, furthermore, how these powerful therapeutic tools can be used to obtain substantial remissions on multiple occasions, if needed, in the same patient. Thus even short term therapies can produce long term remissions.

In the six patients with ANCA positive vasculitis the rationale for the use of anti T cell therapy lay in the latter's potential for interfering with T cell control of B cell

responsiveness. The measurement of ANCA in these patients provided a reflection of this and a useful guide to treatment efficacy. In the future the rate of rise of ANCA after short courses of plasma exchange might be a valuable indicator of the activity of the humoral immune response and the need for further therapy. However, for the majority of patients with ANCA associated vasculitis, cyclophosphamide and steroids work well in controlling the response and disease activity, so that monoclonal antibody therapy should be reserved for patients who become intolerant of drug treatment or who develop unacceptable side effects from it.

Despite the fact that all the patients with T cell associated vasculitis had been heavily immunosuppressed, none of these, nor any of the patients with autoantibody associated vasculitis, developed a systemic opportunistic infection. Only Patient 4 developed a localised oesophageal candidiasis, which responded to appropriate anti fungal treatment: furthermore, this patient had previously received total lymph node irradiation and OK T3 therapy as well as the immunosuppressive drugs listed above, prior to CAMPATH therapy. The incidence of routine bacterial and viral infections did not appear to be more frequent nor greater in severity than in other patients similarly immunosuppressed

for other reasons. No patient developed a lymphoma, a recognised complication of prolonged immunosuppression, although Patient 1 died from a carcinoma of the colon, which is not thought to be related to such treatment.

Although theoretically a risk for each patient, only patient 2 has so far developed an anti-idiotypic response. At the time of her early treatment CAMPATH 1H was the only monoclonal antibody available. This precluded the use of anti CD4 antibodies which may, as suggested in animal studies, enhance tolerance to the concomitant use of CAMPATH [31] 1H. Our current policy is to use CAMPATH 1H as the first therapeutic agent and to use anti CD4 as adjunctive therapy if CAMPATH 1H proves to have only a temporary effect. Whether or not this will be successful in preventing further anti-idiotypic responses remains to be seen: in any event the use of plasma exchange to remove circulating anti-idiotypic antibodies prior to monoclonal antibody therapy in Patient 2 has proved to be successful at the time of two subsequent relapses.

The systemic vasculitides are diseases which untreated carry a high morbidity. We have found that humanised monoclonal antibody therapy appears to provide an alternative to

conventional regimens incorporating steroids and cytotoxic agents both of which themselves contribute a separate and substantial iatrogenic morbidity. As the growing understanding of the pathogenesis of the vasculitides points to autoimmune mechanisms, the introduction of specific immunotherapy offers the opportunity for safer and more selective treatment than available hitherto. Our experience of using humanised monoclonal antibodies with specificity for lymphocytes, particularly T cells, suggests that this might be such a suitable strategy, not only for patients with refractory vasculitis, but also might usefully be considered in place of conventional therapy for the treatment of vasculitis at presentation.

ACKNOWLEDGEMENTS

CAMPATH-1H is a trademark of Wellcome plc.

LEGENDS TO TABLES AND FIGURES

Legend to Table 1:

Note all patients with T cell associated vasculitis received more than one course of treatment. with patient 2 receiving a total of five courses to December 1993. Most patients received monoclonal antibody (anti CD52) therapy as an initial dose (2mg) intravenously followed by an increase to 40 mg/d which empirically had been found to produce optimal clinical effect.

Legends to Figures:

Figure 1 Deterioration in creatinine clearance and KCO (pulmonary gas transfer corrected for alveolar volume) values in patient 3 during the four year illness prior to monoclonal antibody therapy and improvement to normal range for both after this treatment. The predicted normal range for creatinine clearance was greater than 70 mls/min and for KCO greater than 1.7. Note transience of lymphocyte depletion and improvement in creatinine clearance after lower dose treatment (up to 10 mg daily) and the sustained recovery of both. as well as KCO. after the higher dose was used (40 mg daily).

Figure 2 Development of antiidiotypic responses to CAMPATH-1H in patient 2. Three months after completion of the second course of CAMPATH 1H a third course was given accompanied for the first time by anti CD4 therapy. No therapeutic effect was observed. An antiidiotypic antibody was detected which was capable of blocking the effect of CAMPATH-1H in vitro. This was effectively removed by intensive daily plasma exchange (PE) and response achieved with monoclonal antibody (mAb) therapy given immediately afterwards.

Figure 3 Effect of CAMPATH-1H therapy, prednisolone and cyclophosphamide on ANCA levels in patient 6. This patient had already received prednisolone (P) 60 mg/day and cyclophosphamide (C) 3 mg/kg/day for two weeks before referral (Day 0). Plasma exchange (arrowed) revealed ANCA synthesis was sustained until the CAMPATH 1H dose was increased to 40 mg daily.

Figure 4 Effect of CAMPATH-1H alone on progression of lung lesions in patient 9. Note increasing size of right upper lobe lesion from June 1992 to February 1993 which cavitates. Left upper and lower lobe lesions are also present by February 1993. Treatment with CAMPATH 1H alone from February 1993 is followed by disappearance of the left lung lesions and by May 1993 there is only segmental scarring in the right upper lobe.

Figure 5 Effect of CAMPATH-1H combined with anti CD4 in patient 11 on circulating anti MPO antibody titres.

Figure 6 Effect of monoclonal antibody therapy on peripheral CD4 cells in patients with T cell associated vasculitis (patients 1-4).

TABLE 1: Details of date and treatment with monoclonal antibody therapy

Patients	Treatment Date	Total Dose mg (number of days x mg/day) CAMPATH 1H		Anti CD4	Remission months
T cell associated					
Autoantibody negative					
1	Nov 1988	28	(8x2.3x2.3x2)	-	0.3
	Feb 1989	6	(3x2)	240 (12x20)	48
	Mar 1993	132	(2.10.3x40)	100 (5x20)	3+
2	Dec 1990	35	(5x2.5x10)	-	7
	Aug 1991	240	(5x8. 10x20)	-	3
	Mar 1992	60	(5x12)	200 (10x20)	12
	Feb 1993	150	(10.20.3x40)	100 (5x20)	8
	Dec 1993	200	(1x200)	100 (5x20)	3
3	Mar 1991	60	(5x2.5x10)	-	
	Apr 1991	200	(5x40)	-	30
4	May 1991	70	(5x2.5x10)	-	3
	Mar 1992	148	(4x12.5x20)	100 (5x20)	0
5	Jul 1992	260	(5x2.5x10.5x40)	-	3
	Nov 1992	200	(5x40)	100 (5x20)	2
	Jan 1993	200	(5x40.3x10)	80 (4x20)	8
ANCA associated					
6	Jul 1992	356	(2.10x4:10.20.7x40)	-	6‡
7	Sep 1992	200	(5x40)	100 (5x20)	14
8	Oct 1992	126	(40.40.10.16.20)	20 (1x20)*	
9	Feb 1993	134	(4.10.3x40)	-	10
10	Sep 1991	60	(5x2:5x10)	-	12
	Mar 1993	132	(2.10.3x40)	100 (5x20)	5
11	Mar 1993	134	(4.10.3x40)	-	1.5
	Jun 1993	134	(4.10.3x40)	100 (5x20)	6

+died ca colon *died cerebrovascular accident at day 7
‡ seroconversion to anti Pr3 positivity. WG asymptomatic

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REFERENCES

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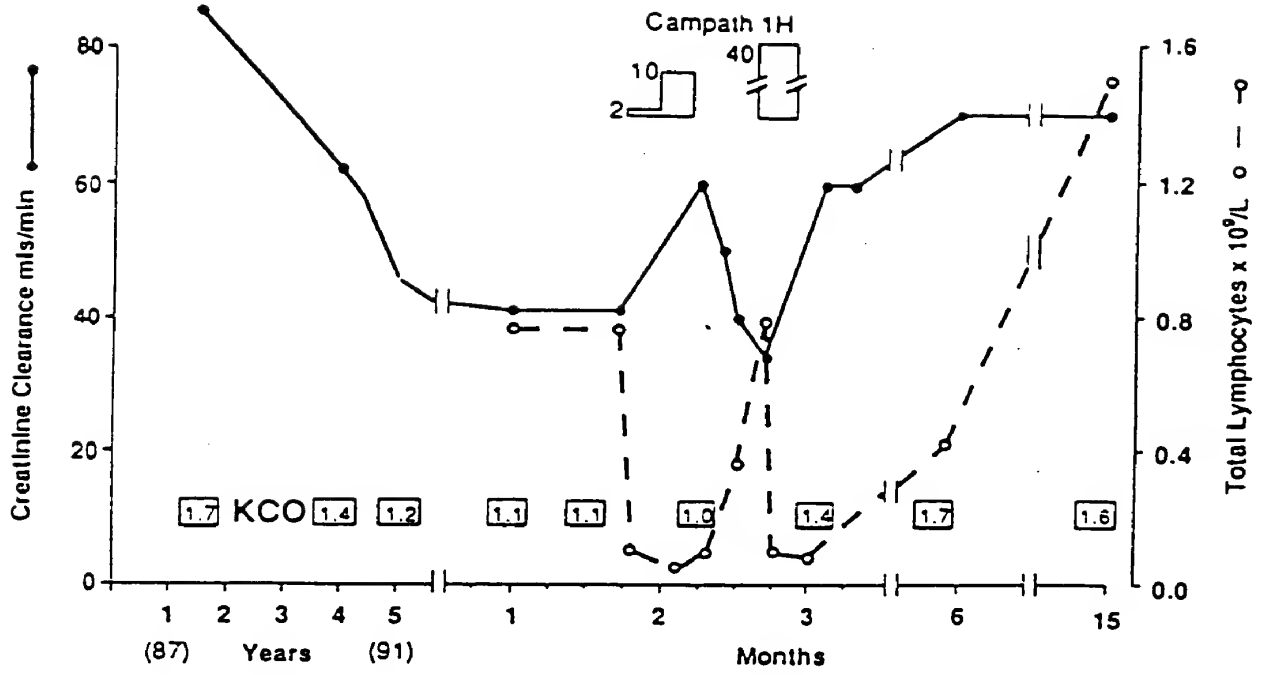
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2
40
Creatinine Clearance mls/min
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Years
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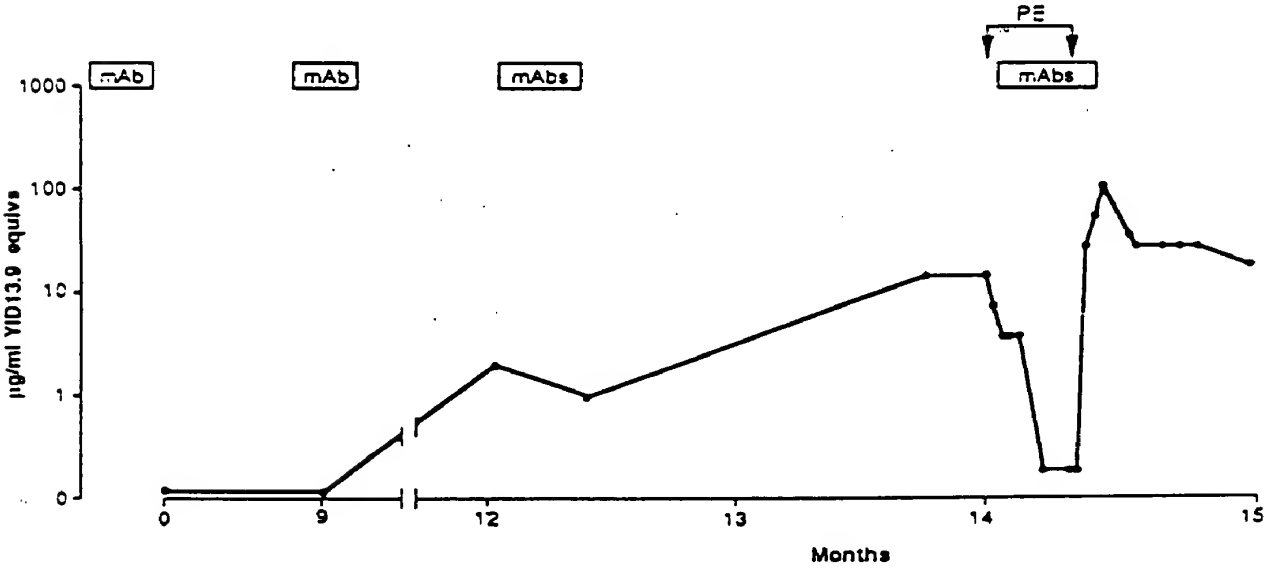
Months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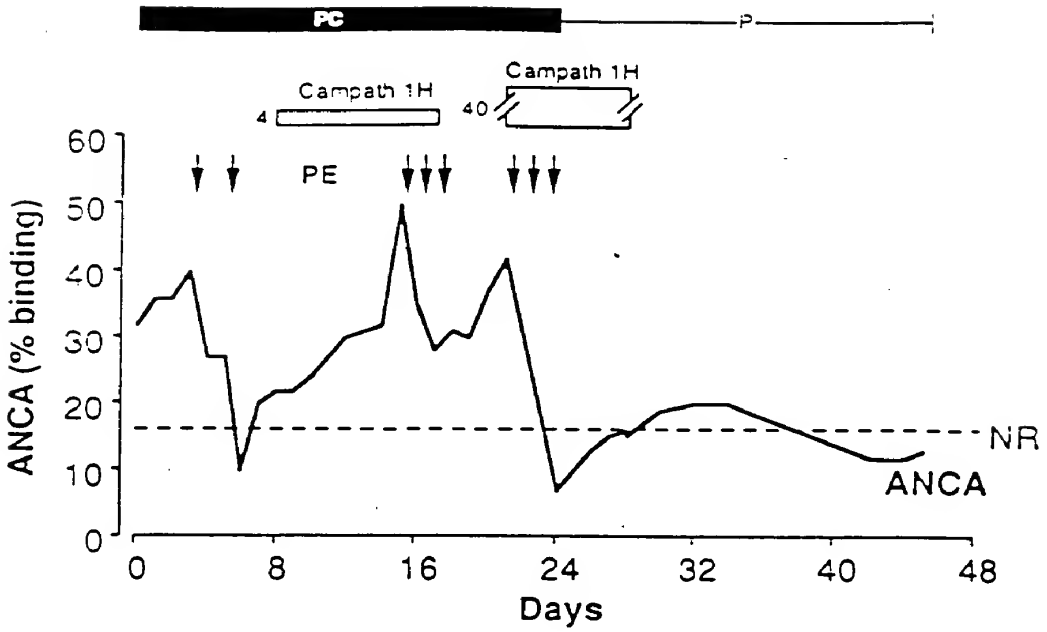
PC
P
Campath 1H
4
Campath 1H
40
PE
ANCA (% binding)
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50
40
30
20
10
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NR
ANCA
0
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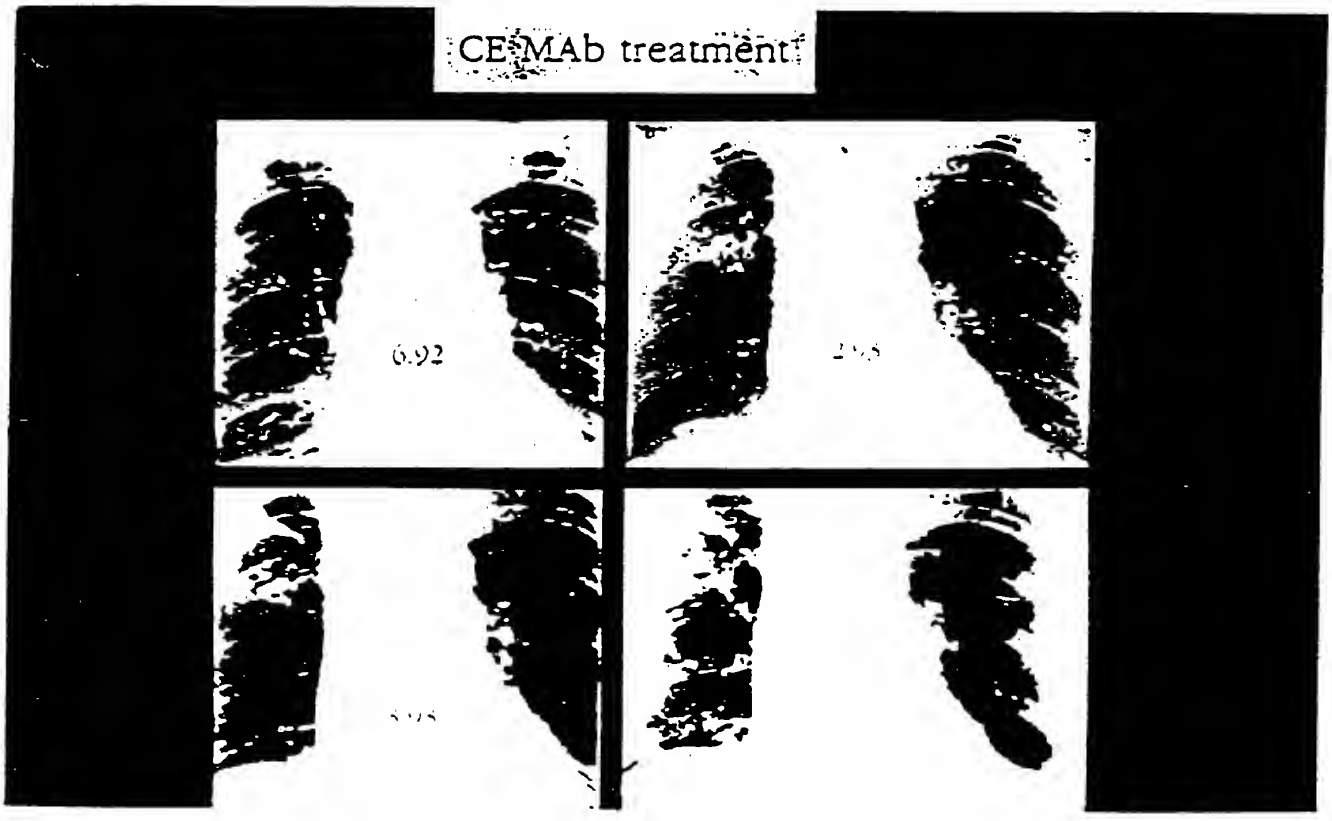
Days


CE MAb treatment
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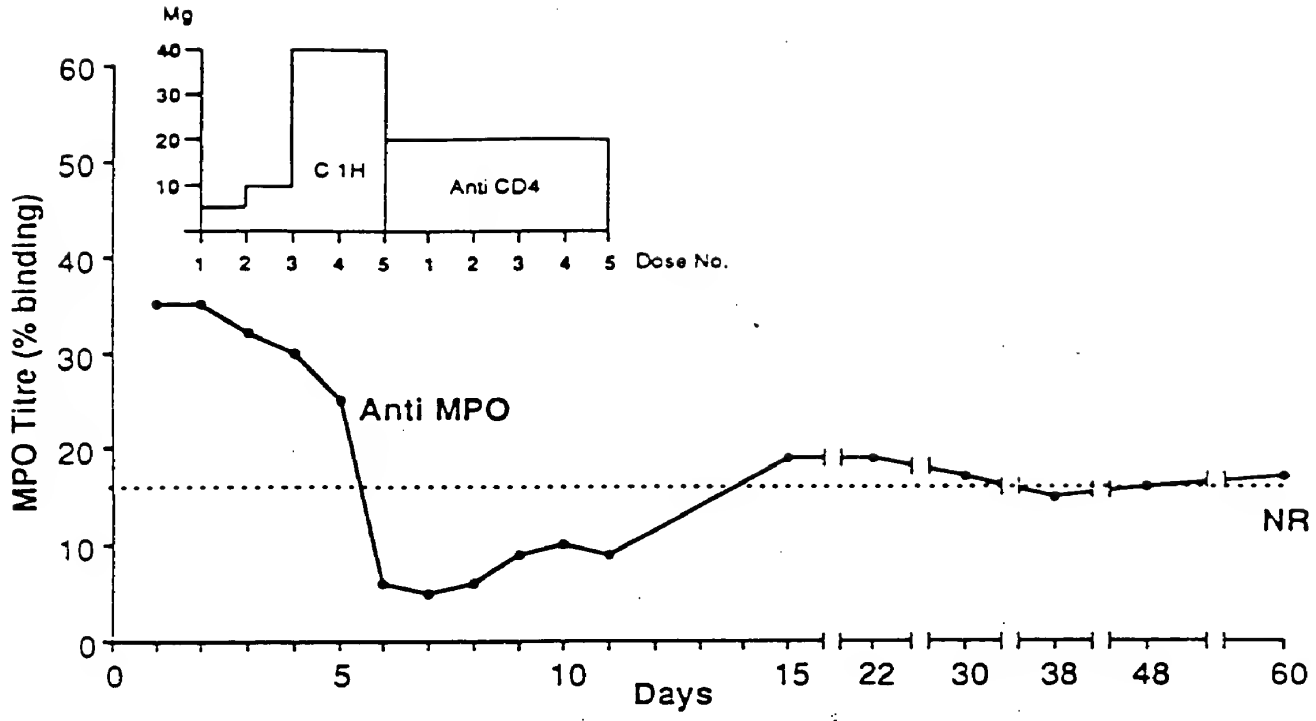
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C 1H
Anti CD4
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Anti MPO
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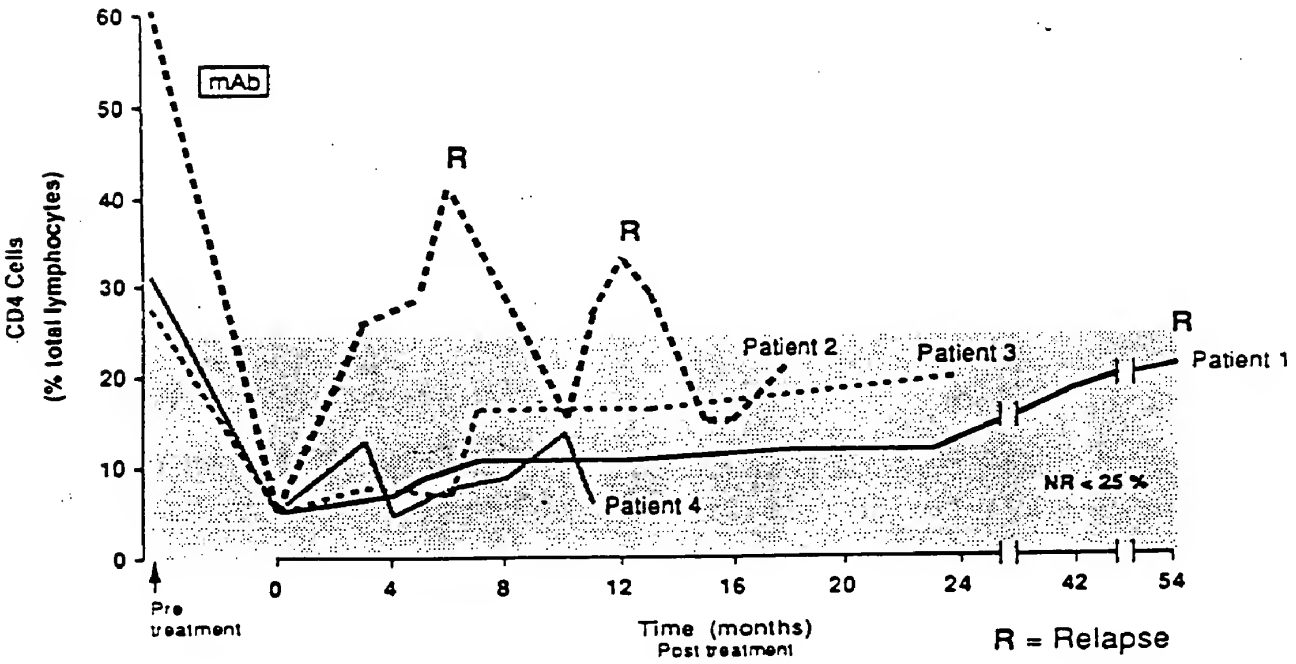
Days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(% total lymphocytes)
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50
40
30
20
10
0
mAb
R
R
R
Patient 2
Patient 3
Patient 1
Patient 4
NR < 25 %
Pre treatment
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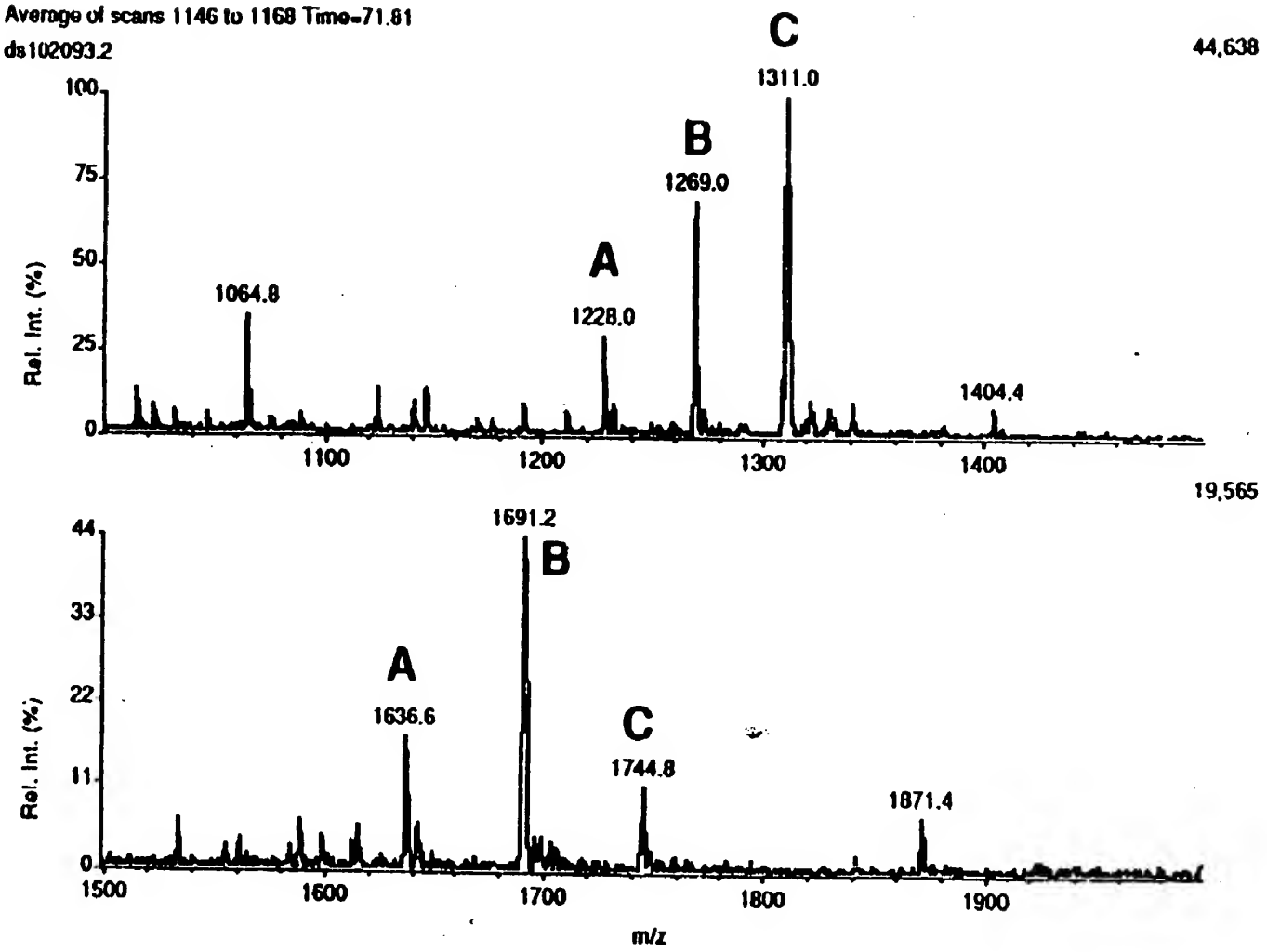
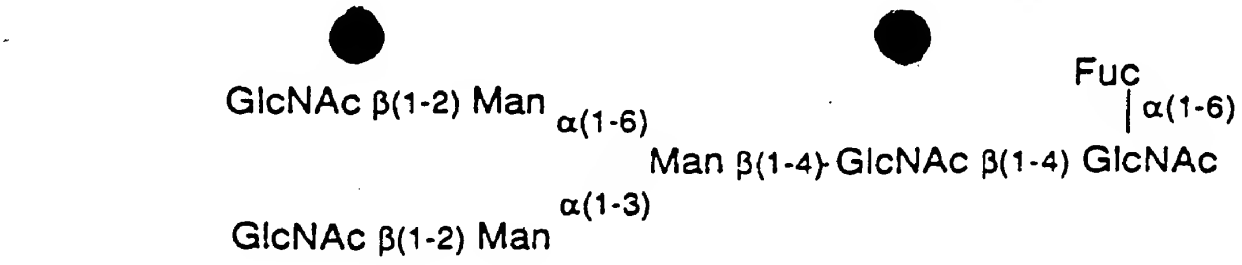
Time (months)
Post treatment
R = Relapse



ES-MS spectrum of the glycosylated peptide of CHO-derived IgG_1. The mass spectrum is shown from m/z 1000 to 2000, split into two ranges. On the top axis are three main peaks corresponding to the +4 charge state of the three major glycoforms of the peptide. On the bottom axis are the peaks corresponding to the +3 charge state. Ions corresponding to glycoforms are indicated.

JSC 2



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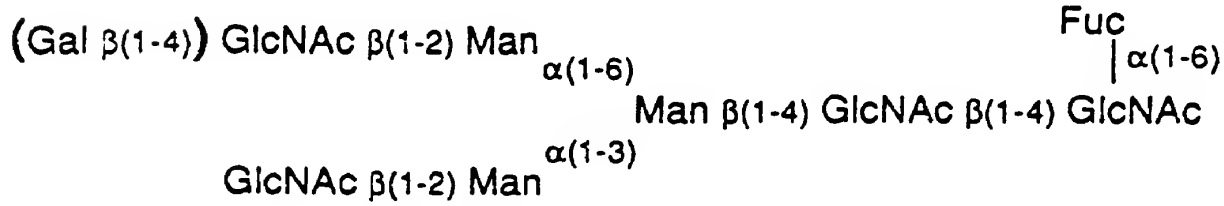

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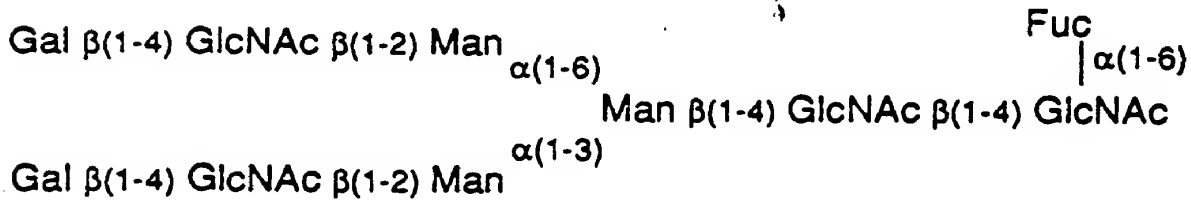
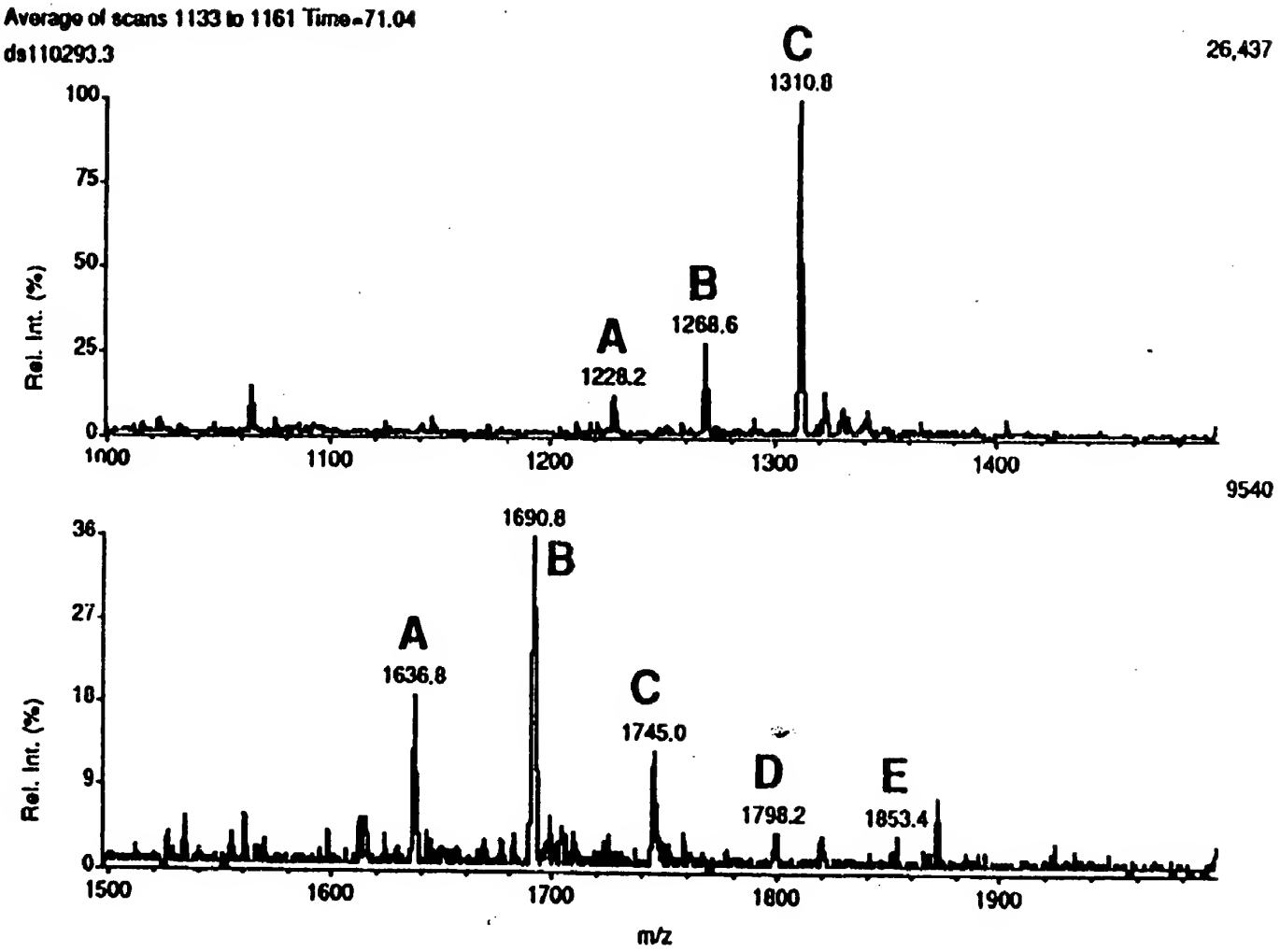


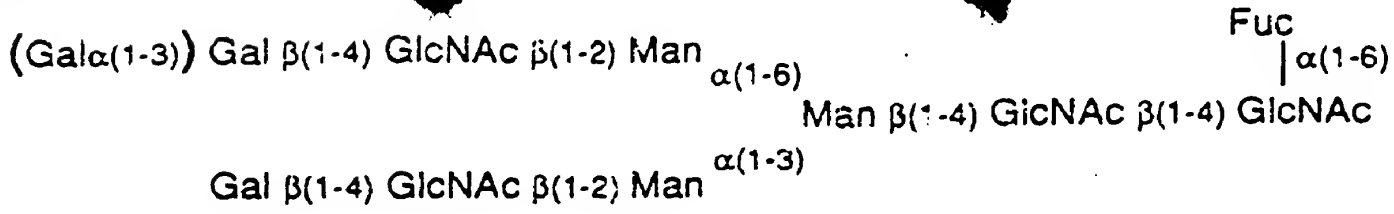
C

Possible structures, inferred from molecular weights, for the oligosaccharides of CHO-derived IgG_1.



ES-MS spectrum of the glycosylated peptide of NS0-derived IgG_1. Ions corresponding to glycoforms have been indicated.

JSC 4



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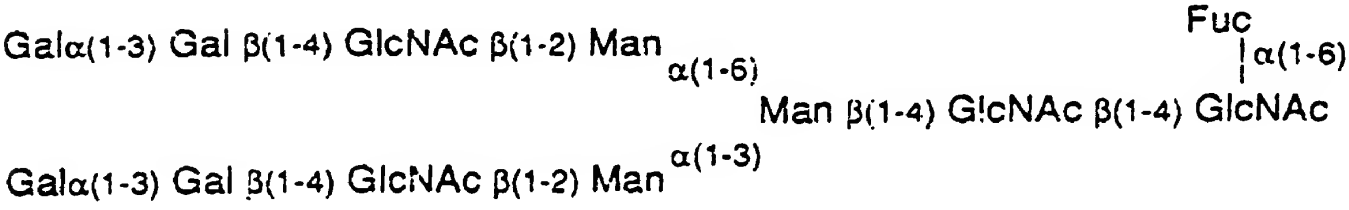

E

Additional structures, inferred from molecular weights, for Galα(1-3)Gal-containing oligosaccharides of NS0-derived IgG_1.